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Research Report

Calmodulin-dependent regulation of neurotransmitter release differs in subsets of neuronal cells

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ABSTRACT

The purpose of this study was to determine whether calmodulin (CaM) plays a role in neurotransmitter release by examining the effect that ophiobolin A (OBA), a CaM antagonist, on neurotransmitter release from clonal rat pheochromocytoma PC12 cells, primary cortical neurons, and primary cerebellar granule cells. OBA inhibited Ca^{2+}/CaM-dependent phosphorylation of cAMP response element binding protein in all cell types tested. Moreover, Ca^{2+}-dependent release of dopamine and acetylcholine from PC12 cells were remarkably reduced by OBA in a dose-dependent and temporal manner, but neurotransmitter release partially recovered with the addition of CaM in membrane permeabilized PC12 cells. OBA and several synthetic CaM antagonists suppressed Ca^{2+}-dependent glutamate release from cerebral cortical neurons, but not from cerebellar granule cells. Myosin Va, a CaM binding protein, localized to synaptic vesicles of PC12 cells and cerebral cortical neurons, but not in cerebellar granule cells. OBA suppressed Ca^{2+}-induced myosin Va dissociation from secretory vesicles, and inhibited secretory vesicle motility in PC12 cells. These results

Abbreviations: ACh, acetylcholine; BSA, bovine serum albumin; CaM, calmodulin; CREB, cAMP response element binding protein; DA, dopamine; DMEM, Dulbecco's modified Eagle's medium; DNase, deoxyribonuclease; FCS, fetal calf serum; Glu, glutamate; hGH, human growth hormone; HRP, horseradish peroxidase; MEM, minimum essential medium; OBA, ophiobolin A; PAGE, polyacrylamide gel electrophoresis; PBS, Dulbecco's phosphate buffered saline; SDS, sodium dodecyl sulphate; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble N-ethylmaleimide-sensitive fusion protein receptor; VAMP-2, vesicle-associated membrane protein-2

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suggest that CaM, although not essential, regulates neurotransmitter release in a subset of neurons and secretory cells, and myosin Va is a possible target of OBA in this process.

1. Introduction

Synaptic transmission is mediated by neurotransmitters released from nerve terminals. Neurotransmitters are stored in synaptic vesicles and released into the synaptic cleft by exocytosis of vesicle contents, which involves docking and fusion of the vesicle membrane with the presynaptic plasma membrane (Jahn and Fasshauer, 2012; Südhof and Rizo, 2013). Recent studies have shown that soluble N-ethylmaleimide-sensitive fusion protein receptor (SNARE) proteins, including syntaxin 1 and synaptosomal-associated protein of 25 kDa (SNAP-25) in the plasma membrane and vesicle-associated membrane protein-2 (VAMP-2, also called synaptobrevin 2) in the synaptic vesicle membrane, play essential roles in neurotransmitter release (Jahn and Scheller, 2006; Hussain and Davanger, 2011; Rizo and Südhof, 2012).

Neurotransmitter release is triggered by Ca^{2+}, and C2-domain containing proteins including synaptotagmin are believed to function as Ca^{2+} sensors for membrane fusion (Pang and Südhof, 2010; Walter et al., 2011). Accumulating evidence suggests that there are several steps prior to membrane fusion and that some of these steps are likely regulated by Ca^{2+}. However, Ca^{2+} sensor proteins regulating these steps are not well understood (Neher and Zucker, 1993; Neher and Sakaba, 2008). Calmodulin (CaM) is the most ubiquitous Ca^{2+} binding protein in cells and has four EF-hand motifs with high Ca^{2+} affinity (Jurado et al., 1999). SNARE-mediated vacuole fusion in yeast is activated by Ca^{2+} efflux from the vacuole lumen and uses CaM as a Ca^{2+} sensor (Peters and Mayer, 1998). CaM is also essential for Ca^{2+}-dependent exocytosis in *Paramecium* (Kerboeuf et al., 1993). In mammalian cells, vesicular Ca^{2+} and CaM have been implicated in intra-Golgi and endosome fusion (Colombo et al., 1997; Porat and Elazar, 2000; Pryor et al., 2000). Early studies using CaM inhibitors and anti-CaM antibodies have demonstrated the involvement of CaM in neurotransmitter and hormone release in mammalian neurons and endocrine cells (Baker and Knight, 1981; Burgoyne et al., 1982; Kenigsberg et al., 1982; Wilson and Kirshner, 1983; Sasakawa et al., 1983; Burgoyne and Norman, 1984; Brooks and Treml, 1984; Kenigsberg and Trifaro, 1985; Ahnert-Hilger and Gratzl, 1987; Matthies et al., 1988; Courtney et al., 1991; Reig et al., 1993; Hens et al., 1996; Igarashi and Watanabe, 2007). In past studies, CaM was identified as a candidate Ca^{2+} sensor for membrane fusion (Okabe et al., 1992; Chamberlain et al., 1995; Kibble and Burgoyne, 1996; Chen et al., 1999). However, this hypothesis has been refuted, since (1) the off-rate for Ca^{2+} dissociation is too slow to account for the transient nature of the response to elevated Ca^{2+} in nerve terminals, and (2) CaM does not bind Ba^{2+}, which could substitute for Ca^{2+} in triggering regulated exocytosis (Burgoyne and Clague, 2003). From other recent studies, it is currently thought that CaM plays a regulatory role, not in the fusion step, but rather in the steps prior to Ca^{2+}-induced membrane fusion (Sakaba and Neher, 2001; Junge et al., 2004; Neher, 2006; Zikich et al., 2008). In the calyx of Held synapse, CaM promotes refilling of the rapidly releasing synaptic vesicle pool. In another study, CaM functioned as a regulator acting antagonistically to synaptotagmin in SNARE-mediated membrane fusion (Di Giovanni et al., 2010).

A number of possible candidates exist for CaM binding proteins involved in CaM-dependent regulation of neurotransmitter release, including Ca^{2+}/CaM-dependent protein kinase II (Ohyama et al., 2002; Nomura et al., 2003), calcineurin (Hens et al., 1998), actin (Sullivan et al., 2000), synaptotagmin (Perin, 1996; Fournier and Trifaro, 1988), Munc13 (Junge et al., 2004; Zikich et al., 2008), Myosin V (Watanabe et al., 2005), syntaxin 1 (Di Giovanni et al., 2010), VAMP-2 (Quetglas et al., 2000, 2002), and rab3A (Park et al., 1997; Coppola et al., 1999). Given the variation in exocytic control mechanisms between adrenal chromaffin cells and glutamatergic synapses (Neher, 2006), CaM-mediated regulation of exocytotic release appears to differ considerably between neurons and secretory cells. In the present study, we examined the effect of ophiobolin A (OBA), a potent CaM inhibitor (Au et al., 2000), on neurotransmitter release in PC12 cells, cerebral cortical neurons, and cerebellar granule cells *in vitro*. Our results reveal striking differences on how OBA regulates these cells, and that OBA may exert its inhibitory effect through the suppression of myosin Va dissociation from secretory vesicles.

2. Results

2.1. *OBA suppresses CaM-dependent CREB phosphorylation in vitro*

Ca^{2+}/CaM-dependent protein kinase IV phosphorylates cAMP response element binding protein (CREB) at Ser^{133}. To assess the effective inhibitory concentration of OBA on PC12 cells and neurons, the effect of OBA on Ca^{2+}/CaM-dependent CREB phosphorylation was examined by Western blot analysis. As shown in Fig. 1, ionomycin treatment markedly enhanced CREB phosphorylation in PC12 cells, cerebral cortical neurons, and cerebellar granule cells. This effect was suppressed by pretreating cells with 3 μM OBA for 30 min. This suggests that OBA effectively inhibited ionomycin-induced CREB phosphorylation in PC12 cells and neurons at a concentration of 3 μM.

2.2. *OBA suppresses both ACh and DA release from PC12 cells*

PC12 cells have two morphologically distinct types of secretory vesicles: one is a large dense-core vesicle containing dopamine (DA) and the other is a small synaptic microvesicle containing acetylcholine (ACh). OBA suppresses exocytotic release of exogenously expressed hGH in the micromolar range (Quetglas et al., 2002). To determine whether OBA